Journal of Clinical Periodontology
Official Journal of The European Federation of Periodontology
Founded by the British, Dutch, French, German, Scandinavian, and Swiss Societies of Periodontology

J Clin Periodontol 2008; 35: 897–905 doi: 10.1111/j.1600-051X.2008.01306.x

Improvement of periodontal condition by probiotics with *Lactobacillus salivarius* WB21: a randomized, double-blind, placebo-controlled study

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Shimauchi H, Mayanagi G, Nakaya S, Minamibuchi M, Ito Y, Yamaki K, Hirata H. Improvement of periodontal condition by probiotics with Lactobacillus salivarius *WB21: a randomized, double-blind, placebo-controlled study. J Clin Periodontol 2008; 35: 897–905. doi: 10.1111/j.1600-051X.2008.01306.x.*

Abstract

Aim: This randomized clinical study was designed to evaluate the effect of probiotic intervention using lactobacilli on the periodontal condition of volunteers without severe periodontitis.

Material and Methods: Freeze-dried *Lactobacillus salivarius* WB21 (WB21)-containing tablets or a placebo were given to volunteers in a double-blind randomized study. A total of 66 volunteers were finally enrolled and randomly assigned to receive tablets containing WB21 (6.7×10^8 CFU) with xylitol or xylitol alone (placebo) three times a day for 8 weeks. Periodontal clinical parameters and whole saliva samples were obtained at baseline (BL), 4 weeks, and the end of the interventional period (8 weeks). Salivary lactoferrin (Lf) levels were measured by enzyme-linked immunosorbent assay. Lactobacilli in saliva and plaque samples was detected by semi-quantitative RT-PCR using 16S rRNA primers.

Results: Periodontal clinical parameters were improved in both groups after an 8-week intervention. Current smokers in the test group showed a significantly greater improvement of plaque index and probing pocket depth from BL when compared with those in the placebo group. Salivary Lf level was also significantly decreased in the test group smokers.

Conclusion: Our results indicate that probiotics could be useful in the improvement/maintenance of oral health in subjects at a high risk of periodontal disease.

Key words: healthy adults; *Lactobacillus salivarius*; periodontal condition; placebo-controlled; probiotics; randomized clinical trial

Accepted for publication 7 July 2008

Conflict of interest and source of funding statement

Two of the authors (S. Nakaya and H. Hirata) were workers of Wakamoto Pharmaceutical Co. None of the other authors declare any potential conflicts of interest.

Financial support for this study was provided by Wakamoto Pharmaceutical Co., Tokyo, Japan; other external funding was not used.

Probiotics are defined as bacteria with physiological benefits for humans and their classification requires (1) scientifically demonstrated beneficial physiologic effects; (2) human origin, safety for human use, and stability in acid and bile; and (3) adherence to intestinal mucosa (Salminen et al. 1998). Probiotics favourably influence both the development and stability of microbiota, thereby inhibiting the colonization of pathogens, enhancing the mucosal barrier via tropic effects on the epithelium, and stimulating both the innate and the adaptive immune systems (Tlaskalová-Hogenová et al. 2004). The most commonly used and studied genera fulfilling the above criteria are *Lactobacillus* and *Bifidobacterium* (Isolauri 2001). Lactobacilli can produce different antimicrobial components including organic acids, hydrogen peroxide,

low-molecular-weight antimicrobial substances, bacteriocins, and adhesion inhibitors (Silva et al. 1987), and have gained prominence as probiotics. *Lactobacillus salivarius*, a common strain in the mouth, is resistant to gastric acid and is also a therapeutic candidate for gastrointestinal disorders (Dunne et al. 1999, 2001).

The effects of probiotic therapy have been studied extensively in a variety of systemic indications and medical disorders (Broekaert & Walker 2006). Presumably, oral administration of probiotics may also benefit oral health by preventing the growth of harmful microbiota or by modulating mucosal immunity in the oral cavity. Recently, a small number of in vitro and in vivo studies have been performed on the role and effects of probiotics in the mouth. Probiotic strains tested in these studies mainly included various strains of lactobacilli as well as *Bifidobacterium*, *Streptococcus salivarius*, *Propionibacterium freudenreichii*, and *Weissella cibara* (Meurman & Stamatova 2007). When compared with the criteria for probiotics in the gastrointestinal tract, ''oral probiotics'' may need some modification or addition. For instance, oral probiotic bacteria should adhere to and colonize on dental tissue, and should be a part of the biofilm. They should not ferment sugars, which subsequently lowers the pH and is detrimental to dental health. The key issues including definitive criteria for classification have not yet been resolved (Meurman 2005).

In the previous reports, consumption of products containing probiotic lactobacilli was shown to successfully reduce caries risk and the number of mutans streptococci in the oral cavity (Näse et al. 2001, Ahola et al. 2002, Çaglar et al. 2006). A few studies also revealed that probiotic *Lactobacillus* strains were useful in reducing gingival inflammation and the number of black-pigmented rods including *Porphyromonas gingivalis* in the saliva and subgingival plaque (Ishikawa et al. 2003, Krasse et al. 2006, Matsuoka et al. 2006). However, little is known about the effects of probiotics on periodontal health and the microbiota of supra- and subgingival plaque.

To determine the effects of probiotics on oral health promotion, we conducted a double-blind, randomized, placebo-controlled clinical trial in healthy volunteers without severe periodontitis. The specific aim of this study was to evaluate whether the oral administration of probiotic tablets containing *L. salivarius* could change the clinical parameters of periodontal tissues and the expression of salivary inflammatory markers.

Material and Methods

Probiotic product

The study product (Wakamate D®; Wakamoto Pharmaceutical Co., Tokyo, Japan) contained 6.7×10^8 colony-forming units (CFU)/tablet of *L. salivarius* WB21 and xylitol (280 mg/tablet). Strain WB21 is an acid-tolerant lactobacilli isolated from *L. salivarius* WB1004 (Aiba et al. 1998). The study product was tested against a placebo tablet from the manufacturer that contained only xylitol (280 mg/tablet) but was of identical taste, texture, and appearance. The dose was three tablets taken orally every day throughout the test period.

Subjects and randomization

Seventy-one volunteers were recruited from the workers of Wakamoto Pharmaceutical Co. by means of advertisement throughout the factory. Recruitment was carried out from April 2005 to July 2005. Gender, age, and smoking status (never, former, current) were recorded for each subject. The medical and dental records of all the applicants were also checked by a questionnaire. Applicants were outwardly healthy and further confirmed to meet the following criteria: (1) not currently visiting their dentists for treatment; (2) not using probiotic supplements; (3) free from adverse reactions to lactose or fermented milk products; and (4) antibiotics not taken within the last month. Oral examination was performed by four dentists to confirm the lack of severe periodontitis and untreated carious lesions that would require treatment. Those applicants with (1) probing pocket depth (PPD) $\geqslant$ 6 mm of the examined teeth and (2) the presence of tooth with excess mobility and/or abscess formation were considered as having severe periodontitis. Four were excluded, and 67 eligible subjects (58 males and nine females; mean age 44.9 ± 8.3 years, range 32–61 years) were finally allocated to test and placebo groups (Fig. 1). All subjects were assigned to one of two groups randomized by gender, age, and smoking status by one of the authors (H. H.) using a randomization table. The randomization code was held by this person remotely from all assessments and was not broken until completion of data analysis. The randomization was concealed by the use of sequentially numbered, identical-appearing containers containing probiotic or placebo tablets.

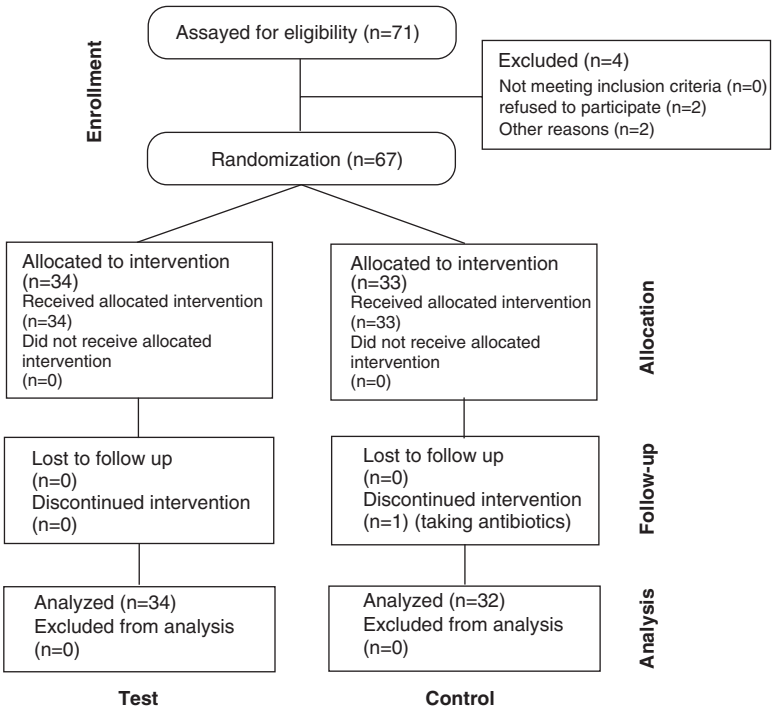

Fig. 1. Flowchart of the subjects in the study.

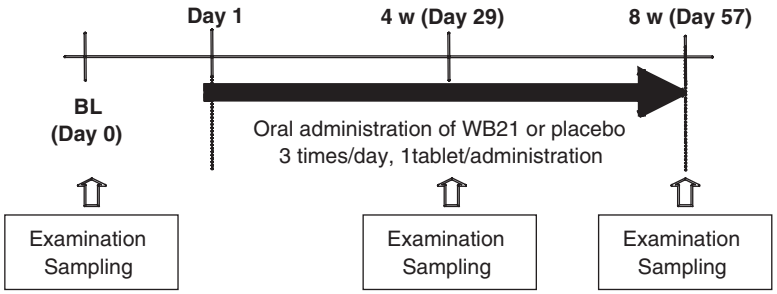

Fig. 2. Experimental schedule.

All subjects provided written informed consent, and the study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry.

Study protocol

The study was performed as a double-blind, randomized, and placebo-controlled design over an 8-week test period (Fig. 2). Those in the test group (WB21 group; $n = 34$) consumed three tablets containing *L. salivarius* WB21 with xylitol (2.01×10^9 CFU/day and 840 mg/day, respectively) every day. Those in the placebo group ($n = 33$) consumed three tablets daily containing xylitol only (840 mg/day). Participants in both groups were directed to place the tablets in the oral cavity for a few minutes, allowing them to dissolve. They were also instructed not to change their oral hygiene regimens and not to take other probiotic products throughout the test period. Neither professional prophylaxis nor tooth-brushing instruction was performed during or before the experimental period. Clinical parameters and saliva/plaque samples were obtained from all subjects on days 0 (baseline; BL), 29 (4 weeks; 4W), and 57 (8 weeks; 8W). The experiment was started in August 2005 and finished in October 2005. One subject was lost in the control group due to consumption of antibiotics during the follow-up period. Finally, 66 subjects, 34 in the test and 32 in the placebo group, were analysed (Fig. 1).

Outcome measures

All clinical measurements were obtained from Ramfjord's six teeth (16, 21, 24, 36, 41, and 44 in the FDI two-digit notation system) in all subjects (Ramfjord 1959) at each visit and considered representative of the whole dentition (Gettinger et al. 1983). PPD was measured using a CP-15 UNC probe (Hu-Friedy, Chicago, IL, USA). Bleeding on probing (BOP; Ainamo & Bay 1975) was assessed. The gingival index (GI; Löe & Silness 1963) was scored according to the modification introduced by Gettinger et al. (1983). Supragingival plaque was scored by plaque index (PlI; Silness & Löe 1964).

All parameters were assessed by four trained clinical investigators experienced with the index systems. When one of the selected teeth was missing in the oral cavity, parameters were obtained from the adjacent tooth in the same area of the jaw.

Saliva and plaque sampling

After the clinical measurements, saliva and plaque samples were obtained. Unstimulated whole saliva was collected into sterile 15-ml plastic tubes. Aliquots were made from saliva samples and stored at -20°C. Sampling sites of the selected six teeth were then isolated with sterile cotton rolls. Supragingival plaque samples were taken with sterile explorers and suspended in 1 ml of distilled water. After thorough removal of supragingival plaque with sterile cotton pellets, three sterile paper points were inserted into the gingival sulcus until resistance was felt. After 30 s, all paper points were removed and pooled in 1 ml of sterile distilled water. Supra/subgingival plaque samples were suspended by vortexing and stored at -20°C until use.

Measurement of lactoferrin levels in saliva

Aliquots of saliva samples were thawed and centrifuged to remove contaminating debris. After filtration through a 0.45 μm filter, the concentration of whole Lf in saliva samples was measured by enzyme-linked immunosorbent assay (EMD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Quantitative detection of *L. salivarius* in saliva and supra/subgingival plaque samples

After thawing, plaque and saliva samples were centrifuged at 9,510 *g* for 5 min. and washed twice with distilled water. After the final wash, bacterial cell pellets were re-suspended in 0.5 ml of distilled water. Bacterial DNA of saliva and plaque samples was extracted and precipitated as described previously (Sakamoto et al. 2001). Briefly, nucleic acids were released by three cycles of freeze-thawing after treatment with enzymes (lysozyme, 5 mg/ml; *N*-acetylmuramidase, 0.1 mg/ml; proteinase K, 2 mg/ml) and sodium dodecyl sulphate (1% w/v). After centrifugation and washing with 70% ethanol, the DNA precipitate was re-suspended in 100 μl of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Real-time polymerase chain reaction (PCR) was performed with an ABI PRISM® 7300 real time PCR system (Applied Biosystems, Foster City, CA, USA). Briefly, amplification was performed on Microamp® optical 96-well reaction plates (Applied Biosystems) in a 50-μl final volume containing 5 μl of template DNA, 25 μl of SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μl each of forward and reverse primer specific for *L. salivarius* (F: CG AAACTTTCTTACACCGAATGC, R: GTCCATTGTGGAAGATTCCC, Byun et al. 2004) or universal primers (F: GTGCTGCACGGCTGTCGTCA, R: A CGTCATCCACACCTTCCTC), and 19 μl of distilled water. Thermal cycling conditions were as follows: 95°C for 10 min., 40 cycles at 95°C for 15 s, and 60°C for 1 min. The C_T value was the cycle in which a statistically significant increase in fluorescence intensity was first detected in association with a logarithmic increase in the PCR product. The detection system constructed a standard curve by plotting the C_T value against each standard dilution. The proportion of *L. salivarius* to total bacteria was calculated by the ratio of fluorescent products produced by the specific and universal primers in each sample.

Statistical analysis

The null hypothesis was that intake of WB21 was no more effective than placebo in improving periodontal clinical parameters. Analysis of gender and smoking status in the test and placebo

groups at BL was performed using the χ^2-test. Other comparisons between both groups regarding age and clinical variables were performed using the Bonferroni *t*-test for normally distributed variables, and the Mann–Whitney *U* or the Wilcoxon signed rank non-parametric test for non-normally distributed or discontinuous variables, as appropriate.

Results

Baseline characteristics of subjects in the test and placebo groups

Baseline characteristics of subjects were similar in test (WB21) and placebo groups (Table 1). No statistical difference was found for any clinical parameter, although WB21 group subjects showed relatively higher PPD and BOP (%).

We further subdivided the subjects into smokers (current) and non-smokers (never/former), and compared the clinical parameters between test and placebo groups (Table 2). No statistical difference was observed between groups in smoker subjects or in non-smoker subjects. However, when smokers and non-smokers were compared within the group, smokers showed a significantly greater PPD, GI, and PlI in the test group, and PPD and GI in the placebo group.

Changes of lactobacilli levels in saliva and supra/subgingival plaque samples

We first compared the proportion of lactobacilli in saliva supra/subgingival plaque samples between test and placebo groups (Fig. 3). The proportion of *L. salivarius* in the saliva tended to decrease during the intervention period for both test and placebo groups. A significant difference between groups was observed at 8 weeks ($p<0.05$; Fig. 3a). In supragingival plaque samples, the proportion was significantly decreased for both groups at 4 and 8 weeks as compared with BL ($p<0.001$, Fig. 3b). The lactobacilli levels in the subgingival plaque of both groups also decreased during the experimental period and were significantly lower at 4 and 8 weeks than at BL ($p<0.01$ and $p<0.001$, respectively; Fig. 3c).

Changes of salivary Lf levels during probiotic intervention

After the 8-week intervention, the salivary Lf level was only significantly decreased in the test group ($p<0.01$, Fig. 4a). We further compared Lf levels in the saliva between both groups when the subjects were divided into current smokers and non-smokers (Fig. 4b and c). Test group smokers showed significantly decreased Lf levels at 8 weeks when compared with the baseline ($p<0.001$), although they tended to have higher Lf levels than the non-smoker controls at BL (Fig. 4b).

Changes of clinical parameters in test and placebo groups during the experimental period

Figure 5 summarizes the changes in clinical parameters (PlI, GI, PPD, and BOP) for the test and placebo groups during the 8-week-period intervention. Both groups showed decreased mean PlI, GI, and BOP at 4 and 8 weeks

Table 1. Demographic and clinical characters of test and control groups

	Test (WB21)	Control (placebo)	*p*-value
No. of subjects	34	32	–
Gender	M29: F5	M28: F4	0.79
Mean age ± SD (Y)	45.0 ± 8.3	44.8 ± 8.4	0.87
Smoking status			
Current	8	7	
Former	12	11	0.98
Never	14	14	
Clinical parameters			
PPD	2.5 ± 0.1	2.4 ± 0.2	0.06
GI	0.8 ± 0.1	0.7 ± 0.1	0.11
BOP (%)	19.2 ± 2.4	13.9 ± 2.5	0.07
PlI	0.7 ± 0.1	0.6 ± 0.1	0.38

PPD, probing pocket depth; GI, gingival index; BOP, bleeding on probing; PlI, plaque index. All clinical parameters are expressed as mean ± SEM.

Table 2. Base characteristics of smokers (current) and non-smokers (never/former) in both the groups

	Test (WB21)		Control (placebo)	
	Smokers ($n=8$)	Non-smokers ($n=26$)	Smokers ($n=7$)	Non-smokers ($n=25$)
PPD	2.9 ± 0.2*	2.4 ± 0.1	2.8 ± 0.7*	2.2 ± 0.1
GI	1.1 ± 0.1*	0.8 ± 0.1	0.9 ± 0.2*	0.6 ± 0.1
BOP (%)	21.8 ± 3.7	18.4 ± 3.0	22.4 ± 9.0	11.8 ± 1.8
PlI	0.9 ± 0.1*	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1

The data are expressed as means ± SEM of each subgroup. The number of subjects in each subgroup was shown in the parenthesis.
*Significantly different ($p<0.05$) from the non-smoker subjects of the same group by Mann–Whitney *U*-test.
PPD, probing pocket depth; GI, gingival index; BOP, bleeding on probing; PlI, plaque index.

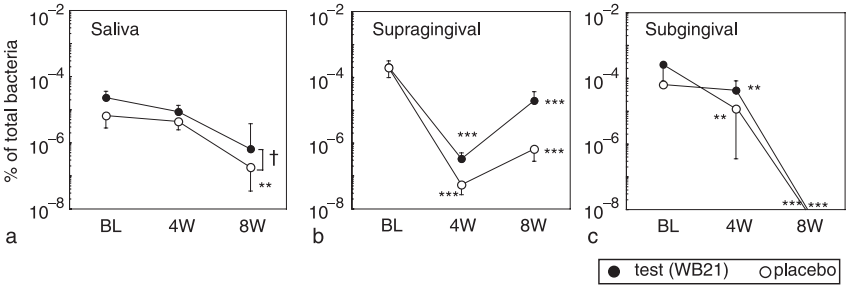

Fig. 3. Prevalence of *Lactobacillus salivarius* in saliva (a), supragingival plaque (b), and subgingival plaque (c) samples from all subjects before and after probiotic intervention. The proportion of *L. salivarius* in the test specimens was determined by real-time polymerase chain reaction. The data are expressed as means ± SEM of each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ by the Wilcoxon signed rank test when compared with the baseline value for each group. †$p<0.05$ by the Mann–Whitney *U*-test when compared with the control group.

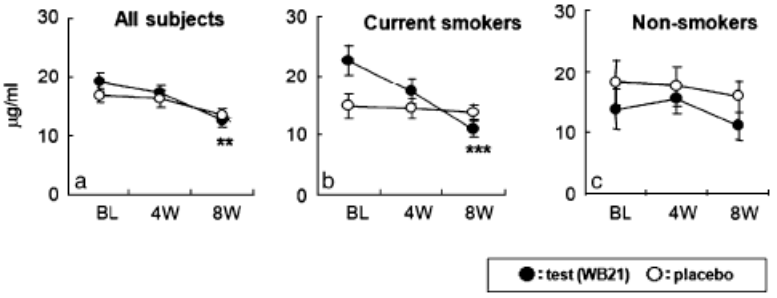

Fig. 4. Changes in salivary Lf levels for all subjects (a), current smokers (b), and non-smokers, i.e. never and former smokers (c) by probiotic intervention. The Lf level in each saliva sample was determined using ELISA in triplicate assays. The data are expressed as the mean ± SEM of each group. $^{**}p<0.01$, $^{***}p<0.001$ by the Wilcoxon signed rank test compared with the baseline value for each group.

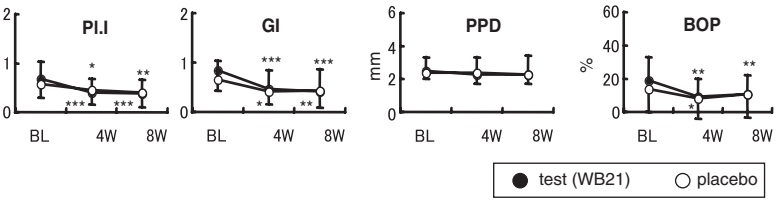

Fig. 5. Changes in periodontal clinical parameters after probiotic intervention. Error bars indicate SDs. $^{**}p<0.01$, $^{***}p<0.001$ by the Wilcoxon signed rank test when compared with the baseline value for each group.

when compared with the baseline regardless of the presence of probiotic lactobacilli. Then, the change of each parameter at 4 and 8 weeks from BL was calculated and compared across all test and placebo groups using the following formula: ΔChange = (value at 4 or 8 week) − (BL value). No statistically significant difference was observed between groups in terms of the Δchange in all clinical indices (Fig. 6a). We then divided the subjects in each group into current smokers and non-smokers and compared the Δchange in clinical parameters. Current smokers in the test group showed a significant decrease in PlI and PPD at 4 and 8 weeks when compared with the placebo group (Fig. 6b). The mean ΔPlI values [95% confidence interval (CI)] for current smokers in the test and placebo groups were − 0.41 (95% CI, − 0.70; − 0.12) and 0.11 (95% CI, − 0.09; 0.32) at 4 weeks ($p<0.01$), and − 0.35 (95% CI, − 0.64; − 0.05) and 0.03 (95%CI, − 0.25; 0.31) at 8 weeks ($p<0.05$), respectively. The mean ΔPPD values in the same group were − 0.34 (95% CI, − 1.00; 0.32) and 0.40 (95% CI, 0.07; 0.73) at 4 weeks ($p<0.05$) and − 0.19 (95% CI, − 0.72; 0.34) and 0.53 (95% CI, − 0.04; 1.10) at 8 weeks ($p<0.05$), respectively. However, no statistical differences were observed between smokers and non-smokers in each group for any clinical parameter (Fig. 6c).

Adverse events

No adverse events were reported, although one subject in the control group smoker was withdrawn during the follow-up period. This subject took antibiotics prescribed by a physician because of respiratory infection after the examination at 4 weeks, and stopped the intake of tablets. However, this was judged not to be an adverse reaction related to the intervention.

We added the data of the withdrawn subject at BL and 4 weeks to run statistics for the secondary analysis. The statistical differences were the same as the data shown in Figs 3–5 and Tables 1 and 2 (data not shown). As the withdrawn subject was a smoker, we then re-calculated the Δchange of clinical parameters at 4 weeks, subdividing both the groups according to smoking status. The same tendency of Δchange as shown in Fig. 6a and b was obtained after including this subject in the placebo group smokers, although the between-group difference was significant for ΔPlI at 4 weeks ($p<0.05$ by the Bonferroni *t*-test). We finally judged that the observed phenomena were the same regardless of the data of the withdrawn subject.

Discussion

Probiotics are often regulated as dietary supplements and marketed for improving or maintaining health (de Jong et al. 2003).The probiotic tablets tested in this study (Wakamate D®; Wakamoto Pharmaceutical Co.) were originally prepared for contributing to the intestinal microbial balance by providing acid-tolerant *L. salivarius* WB21. Using these tablets, we found that orally administered *L. salivarius* WB21 significantly decreased the PlI and PPD of subjects who were smokers, suggesting clinical improvement of the periodontal condition by probiotic intervention (Fig. 6b). A significant difference in salivary Lf levels was also observed for smokers at 8 weeks (Fig. 4b).

The use of probiotics for promoting general health was extensively studied over the past century after Ilya Metchinikof developed the hypothesis that lactic acid-producing bacteria in the gastrointestinal tract could be beneficial for general health (Çaglar et al. 2005). However, less than a decade has passed since probiotics have been extensively investigated from the perspective of oral health, although a very early report appeared in the 1950s (Kragen 1954). The literature from the past few years has shown that probiotic administration effectively reduces the number of *Streptococcus mutans*, suggesting a role for probiotics in caries prophylaxis (Meurman & Stamatova 2007). More recently, Hatakka et al. (2007) reported that probiotics also reduced oral *Candida* counts in the elderly and might offer a new strategy for controlling oral yeast infections. However, only a limited number of studies have examined the effectiveness of probiotics for periodontal diseases. Grudianov et al. (2002) reported that probiotics were effective for normalization of microbiota in periodontitis and gingivitis patients when compared with a control group. Krasse et al. (2006) showed decreased gingival bleeding and reduced gingivitis after the administration of probiotic *Lactobacillus reuteri*. In Japan, two randomized controlled trials (RCTs) also reported the effects of probiotics on periodontal pathogens. Ishikawa et al. (2003) and Matsuoka et al. (2006) reported that oral administration of probiotic tablets

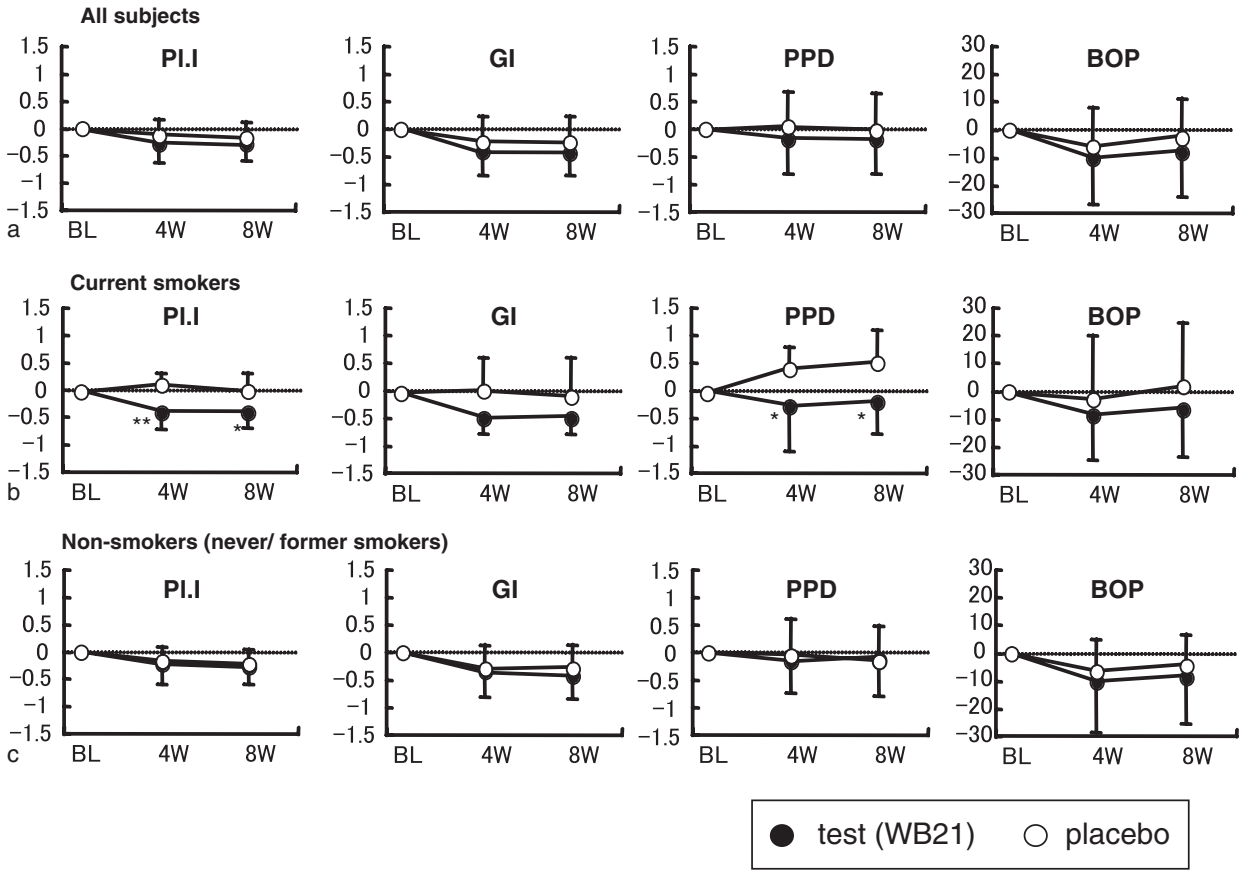

Fig. 6. ΔChange in periodontal clinical parameters for all subjects (a); current smokers (b), and non-smokers, i.e. never and former smokers (c) at 4 and 8 weeks after intervention. The data are expressed as the mean ± SD of the Δchange in the clinical parameters at each indicated time point. $^{*}p<0.05$, $^{**}p<0.01$ by the Bonferroni *t*-test when compared with the control group.

containing *L. salivarius* TI2711 (LS1) to healthy volunteers significantly reduced the number of *P. gingivalis* in the saliva and subgingival plaque, although no significant change was observed in the placebo group. We also investigated the effect of probiotic lactobacilli on oral microbiota in the same subjects and found that oral administration of probiotic lactobacilli successfully reduced the prevalence of periodontopathic bacteria even in non-smokers (unpublished data). Taken together with previous studies, our RCT results suggest that probiotics with lactobacilli may effectively suppress periodontopathic bacteria in the oral cavity and improve the periodontal condition.

We selected *L. salivarius* WB21 as the probiotic strain in the present study, as several strains of lactobacilli have shown potential growth inhibition of *S. mutans* (Meurman & Stamatova 2007). Lactobacilli constitute approximately 1% of cultivable oral microflora (Marsh & Martin 1999), and *L. salivarius* is one of the predominant species in the healthy human mouth (Colloca et al. 2000). Kõll-Klais et al. (2005) reported that the presence of an obligatory homofermentative group of lactobacilli including *L. salivarius* was inversely associated with chronic periodontitis-related clinical parameters and periodontal pathogens. Very recently, Kõll et al. (2008) characterized 22 strains of orally isolated lactobacilli with regard to antimicrobial activities on oral pathogens including periodontopathic bacteria and tolerance to environmental stress in vitro. The majority of strains including *L. salivarius* were shown to suppress the growth of *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *P. gingivalis*, and *Prevotella intermedia*, suggesting a potential for oral lactobacilli to be used as probiotics for periodontal health.

Probiotics using lactobacilli are widely accepted as the bacterial replacement therapy for intestinal microbiota. However, several strains of non-pathogenic or avirulent streptococci produce bacteriocin-like antimicrobial substances and have been nominated for replacement therapy aimed at dental caries, otitis media, and streptococcal pharyngitis (Tagg & Dierksen 2003). Recently, Teughels et al. (2007) reported that the subgingival application of a bacterial mixture including *Streptococcus sanguinis*, *S. salivarius*, and *Streptococcus mitis* after scaling and root planing significantly suppressed the re-colonization of *Porphyromonas gulae* (canine *P. gingivalis*) and *P. intermedia* in a beagle dog model. Therefore, more research is needed to identify appropriate effector strains for oral probiotics specifically designed to prevent and treat periodontal diseases.

It is well known that the normal microbiota in healthy humans display remarkable quantitative and qualitative stability that limits the growth and persistence of interacting microbes. Longer contact between probiotic bacteria and

plaque should increase probiotic activity; thus, in the present study, probiotic tablets were placed in the mouth for a few minutes to allow direct contact. The prevalence of lactobacilli in the saliva was significantly higher in the test group after the intervention (Fig. 3a), which may suggest the translocation of exogenous *L. salivarius*. However, the prevalence of *L. salivarius* in oral specimens from subjects in both groups tended to decrease during the intervention period. When considering the therapeutic application of probiotics to periodontal disease, total removal of plaque seems to be an important step for upsetting the equilibrium and enhancing the replacement of indigenous microbiota. However, it should be noted that this study only aimed to assess the ability of probiotic supplements to promote and maintain periodontal health in healthy subjects. The use of prebiotics or synbiotics may be an alternative approach for increasing the chance of superinfection by probiotics (Tuohy et al. 2003). The probiotic tablets used in this study contained xylitol as an integrant. In preliminary experiments, we observed that addition of conditioned media from WB21 cells suppressed the growth of several periodontal pathogens including *P. gingivalis*, and that xylitol synergistically enhanced the inhibitory effect. However, xylitol alone did not stimulate the growth of *L. salivarius* WB21 or inhibit the growth of periodontopathic bacteria in vitro (data not shown). Klewicki & Klewicka (2004) reported that the growth of lactic acid bacteria was weak or there was no increase in biomass in the presence of polyols including xylitol, suggesting that lactobacilli cannot metabolize xylitol. Galactosylated polyol compounds were metabolized by *Lactobacillus* spp. and enhanced the suppression of pathogenic bacteria in the same study. Further studies are necessary to determine the most suitable prebiotics for developing oral probiotics.

In this study, a significant reduction of clinical indices was also observed in placebo group subjects (shown in Fig. 5). We considered it unlikely that this phenomenon was induced by xylitol in the placebo tablets because of the results of our preliminary experiments. In addition, Matsuoka et al. (2006) also observed improved clinical symptoms in a placebo group administered non-probiotic tablets without xylitol. It is more likely that an attention bias (a Hawthorne effect) occurred within our subjects. The experimental protocol did not include any oral hygiene instruction before/at baseline; however, subjects in both groups might have systematically altered their oral hygiene regimens due to observation. Many factors including attention bias contribute to perceived placebo effects in clinical trials, even in large-scale placebo-controlled RCTs (Ernst 2007). A possible Hawthorne effect was also suggested in clinical studies of periodontal treatment and glycaemic control in diabetic patients (Watts 2006). To avoid this bias, we decided to calculate the Δchange from the BL value of each parameter and compare values between the groups.

Smokers in the test group showed a significantly greater reduction of PlI and PPD at 4 and 8 weeks when compared with the placebo group (Fig. 6b). However, statistical differences between groups were not observed for the total subject pool (smokers and non-smokers) or non-smokers alone (Fig. 6a and c). Smoking is recognized as a major risk factor and a strong predictor for the incidence and progression of periodontitis (Heitz-Mayfield 2005). In this regard, Bergström et al. (2000) reported an increase in the number of periodontally diseased sites and amount of bone loss in current smokers as opposed to non-/former smokers whose periodontal condition remained stable in a 10-year prospective study. All our enrolled subjects never had severe periodontitis; however, current smokers had a significantly worse periodontal condition in both groups, suggesting a generally worse periodontal condition when compared with non-smokers (Table 2). This deteriorated condition may have permitted significant clinical improvement by probiotics. As shown in Fig. 4b, smokers in the test group showed significantly decreased salivary Lf after 8 weeks of intervention. Lf is a member of the transferring family of iron-binding proteins that have antimicrobial properties in most exocrine secretions (Weinberg 2001). It has been reported that the concentration of Lf in the gingival crevicular fluid is correlated with clinical parameters of periodontitis (Wei et al. 2004). We have recently shown that the salivary concentration of whole Lf, as well as that of inflammatory Lf-degraded peptides, was associated with periodontal inflammation (Komine et al. 2007). Thus, the significant decrease in salivary Lf may indicate improvement of periodontal inflammation in smokers in the test group. Taken together, our results suggest that a probiotic intervention could be a useful tool for treatment of inflammation and clinical symptoms of periodontitis, especially in high-risk subjects (e.g. smokers).

Limitations of the present study include the relatively small number of participants and that patients suffering from periodontitis were excluded from the study population. Therefore, further studies including a large-scale RCT are necessary to determine the utility of probiotics as an alternative approach for the treatment and prevention of periodontal diseases.

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Clinical Relevance

Scientific rationale for the study: The systemic effects of probiotics have been studied extensively. However, little is known about the utility of probiotics in the field of dentistry, especially for periodontal health.

Principal findings: After a probiotic intervention with *Lactobacillus salivarius* WB21 for 8 weeks in healthy volunteers, the periodontal condition was improved in test and placebo groups. Current smokers in the test group showed significantly greater reduction of plaque index and probing pocket depth than placebo group smokers.

Practical implications: These findings suggest that a probiotic intervention could be a useful tool for the clinical improvement of periodontal condition.